

12

EUROPEAN PATENT APPLICATION

21 Application number: 80300546.1

51 Int. Cl.³: **C 12 N 1/02**
C 12 N 7/02, C 12 Q 1/00

22 Date of filing: 25.02.80

30 Priority: 28.02.79 US 15895

43 Date of publication of application:
01.10.80 Bulletin 80/20

84 Designated Contracting States:
AT BE CH DE FR GB IT LU NL SE

71 Applicant: **NORTHWESTERN UNIVERSITY**
Evanston
Illinois 60201 (US)

72 Inventor: **Widder, Kenneth, Jon**
115, Carol Street
Carrboro North Carolina 27510 (US)

72 Inventor: **Senyel, Andrew Erwin**
14-1/2 38th Place
Long Beach California 90803 (US)

74 Representative: **McVey, Kenneth William Henry**
Erl Wood Manor
Windlesham, Surrey (GB)

54 Method of magnetic separation of cells and the like, and microspheres for use therein.

57 Magnetically-responsive microspheres having Protein A associated with the outer surfaces thereof are reacted with antibodies selective to the cells, bacteria, or viruses to be separated from a mixed population to attach the antibodies in oriented relation with their Fab arms extending outwardly, and the microspheres are then used in a magnetic separation procedure. The preferred microspheres are prepared from a mixture of albumin, Protein A, and magnetic particles, the microspheres being prepared so that the Protein A is present in the exterior surfaces for antibody binding.


EP 0 016 552 A1

METHOD OF MAGNETIC SEPARATION OF CELLS AND THE
LIKE, AND MICROSPHERES FOR USE THEREIN

This invention relates to the fractionation of heterogeneous populations of cells or the like to isolate a relatively homogeneous sub-population of a specific cell type. More specifically, the improvement of this invention relates to magnetic sorting of cells, bacteria, or viruses.

A general procedure for magnetic sorting of cells, bacteria, and viruses is disclosed in United States patent 3,970,518, issued July 20, 1976. In that procedure, uncoated particles of a magnetic material, such as iron oxide, are contacted with a high concentration liquid dispersion of the selective antibody, and after sufficient antibody has adhered to the magnetic particles, the coated particles are contacted with the mixed population to be fractionated, the select cell or the like binding to the magnetic particles, and the bound cells are then separated magnetically from the remainder of the population. As a further step, the select cells may be separated from the magnetic material, by the use of a cleaving agent solution and magnetic removal of the magnetic particles.

While there are literature reports describing the use of magnetic microspheres in cell sorting, there is no literature verification that uncoated magnetic particles can be made to effectively bind with antibodies. In the published procedures, the particles of magnetic material are contained in microspheres formed from polymers, which can be chemically coupled to antibodies. See, for example: Molday et al, Nature, 268, 437 (1977); Kronick et al, Science, 200, 1074 (1978); and Antoine et al, Immunochemistry, 15, 443 (1978). These references




describe magnetically-responsive microspheres formed from acrylate polymers, such as hydroxyethyl methacrylate, or polyacrylamide-agarose microspheres. Such microspheres can be chemically coupled to antibodies with glutaraldehyde or other di-aldehyde. As described by the cited Molday (1977) and Kornick references, one procedure involves the chemical attachment of diaminoheptane spacer groups to the microspheres, which are then chemically linked to the antibodies by glutaraldehyde reaction. Although effective bonding of the antibodies can be obtained, such procedures are difficult since aggregation of microspheres can readily occur and the preparative procedure is time consuming. For example, the reaction to attach spacer groups may require from five to twelve hours of chemical reaction time, and subsequent dialysis to remove the excess reagent. The coupling of the antibodies may then require another twelve to twenty-four hours followed by dialysis to remove excess coupling agent. Further, such antibody reagents may not be used efficiently, since an excess of the antibodies will usually need to be present during the chemical coupling.

Another disadvantage of magnetic particle or microsphere separation methods as described in the art is that the antibodies are attached to the microspheres in a random manner. Antigen-binding occurs through the Fab regions of the antibodies which are in the outer portions of the arms. With random attachment of the antibodies, one or both of the Fab arms may be unavailable for antigen-binding. Thus, an excess of antibody must be used to assure that the coated microspheres effectively bind to the antigens associated with the cells or other bodies being sorted.

The present invention utilizes staphylococcal Protein A to overcome the limitations of prior art magnetic sorting procedures, as described above. It is known that staphylococcal Protein A selectively binds to antibodies through the Fc region of the antibodies, which is located in the tail portions of the antibodies remote from the Fab arms. See Forsgren et al, J. Immunol., 99, 19 (1967). Heretofore, however, this property of Protein A has not been utilized to form magnetic microspheres. Protein A has been coupled to Sepharose beads (cross-linked agarose gels) to provide a column material with immunoglobulin-binding properties. The column may be used for affinity chromatography, for example, of the IgG fraction of serum. Such chromatographic column materials are commercially available.

Protein A has also been used in procedures for cell separation by density gradient centrifugation. See, for example, Ghetie et al, Scand. J. Immunol., 4, 471 (1975). In a typical procedure, sheep erythrocytes are coated with Protein A by CrCl_3 coupling, and the coated erythrocytes are then contacted with mouse lymphocytes which have been previously reacted with antibodies to prepare the cell surfaces for binding to Protein A, thereby resulting in rosetting of the lymphocytes around the erythrocytes. The resulting rosetted cells are recovered by density gradient centrifugation.

In accordance with the present invention, as distinguished from prior art procedures, magnetically-responsive microspheres are prepared having Protein A associated with the surfaces thereof, and the resulting microspheres are first reacted with the select antibodies before the microspheres are used for cell separation.




With the microspheres used in the method of this invention the antibodies are thereby arranged in oriented attachment on their outer surfaces with the Fab arms of the antibodies extending outwardly. The effectiveness of the microspheres for antigen binding and use in magnetic sorting procedures is thereby maximized. This greatly increases the efficiency with which the select antibodies may be used. Further, it eliminates the need for chemical coupling of the antibodies.

In a preferred embodiment, the microspheres are prepared by mixing Protein A with a polymer matrix material which does not mask the antibody-binding sites of the Protein A. The resulting microspheres having the Protein A in the outer surfaces thereof do not require chemical coupling of the Protein A to preformed microspheres. Albumin appears to be a particularly suitable matrix material for preparing such microspheres. When the microspheres are formed from an aqueous admixture of albumin, Protein A, and magnetic particles, the Protein A is effectively available in the outer surfaces of the microspheres, in effect, forming surface layers on the microspheres with the Protein A in high concentration. The explanation for this result is not fully understood, but appears to relate to the wetting agent or surface tension properties of Protein A when dispersed in an aqueous solution in admixture with albumin.

Thus the present invention comprises a method for separation of a select population of cells, bacteria, or viruses from a mixed population thereof, in which the microspheres containing magnetic particles are coated with a layer of antibodies which selectively bind to the select population. The coated microspheres are contacted

with the mixed population, and the bound select population is magnetically separated from the rest of the mixed population. The method improvement is characterized by modifying the surfaces of the microspheres prior to coating them with antibodies to provide staphylococcal Protein A distributed thereover in adherent relation to the microspheres. The microspheres are then contacted with antibodies which bind the Protein A and which also bind selectively to the select population. By this means the antibodies are arranged in oriented attachment on the surfaces of the microspheres with their Fab arms extending outwardly. Thereafter, the rest of the steps of the magnetic separation are carried out, as is known in the art. Preferably, the microspheres are formed of a polymer matrix material in admixture with the magnetic particles and Protein A, such as an albumin matrix material in an amount of 100 parts per 5 to 40 parts of Protein A. Alternatively, however, the Protein A may be chemically-bonded to the exterior surfaces of the microspheres to provide a Protein A coating thereon.

Where chemical coupling procedures are used, the microspheres may be formed from any matrix material which can be chemically coupled to Protein A, including albumin or other amino acid polymer, and synthetic polymers, such as acrylate polymers. For example, the microspheres may be formed from methyl methacrylate, hydroxyethyl methacrylate, methacrylic acid, ethylene glycol dimethacrylate, agarose polymers, polyacrylamide polymers, or mixtures of such polymers. Protein A may be directly coupled to solid support surfaces containing magnetically responsive materials by several procedures. See, for example, Molday et al, J. Cell Biology, 64, 75 (1975)




-6-

Microspheres can be derivatized with either aminocaproic acid or diaminoheptane which provide extended functional groups for coupling proteins to insolubilized matrixes. Alternatively, with solid surfaces already containing functionally available groups (i.e. amino groups on albumin microspheres) a direct glutaraldehyde coupling of Protein A may be accomplished.

An alternate preferred procedure is to incorporate the Protein A in the microspheres by admixing it with the matrix material prior to the formation of the microspheres, and carrying out the preparation so that the Protein A is available in the outer surfaces of the microspheres. Suitable procedures for preparing such microspheres will therefore be described, but it should be understood that the present invention in its broad method aspect is not limited to the use of such preferred microspheres.

For use in the present invention, the Protein A can be prepared from Staphylococcus aureus by procedures described in the literature. See, for example, Forsgren et al, J. Immun., 97, 822 (1966); and Kronvall et al, Immunochemistry, 7, 124 (1970). Staphylococcal Protein A is also available from commercial sources, such as Pharmacia Fine Chemicals, Piscataway, New Jersey.


The preferred matrix material for forming the microspheres by admixture with Protein A is an amino acid polymer, such as albumin. Animal or human albumin may be used, for example, human serum albumin. Other water-soluble proteins can be used such as hemoglobin, or synthetic amino acid polymers including poly-L-lysine and poly-L-glutamic acid.



When the Protein A is premixed with the matrix polymer, and the microspheres formed therefrom, sufficient Protein A should be included so that the outer surfaces of the microspheres will bind antibodies through the selective action of the Protein A. In general, the microspheres may contain from 2 to 40 parts by weight of Protein A per 100 parts of the matrix polymer such as albumin. Preferred proportions are from about 10 to 35 parts of the Protein A per 100 parts of the matrix polymer.

A sufficient amount of finely-divided particles of a magnetic material should also be included so that the microspheres are magnetically-responsive. For example, the magnetic particles may be ferri- or ferro-magnetic compounds, such as magnetic iron oxides. Other useable magnetic materials in particulate form are disclosed in U.S. patent 3,970,518. A preferred magnetic material is magnetite (Fe_3O_4). Depending on the size of the microspheres, the magnetic particles may range in size from 100 to 20,000 Angstroms. The microspheres may contain from 10 to 150 parts by weight of the magnetic material per 100 parts of the matrix polymer. The microspheres may range in size from 0.2 to 100 microns in diameter. Preferably, however, the microspheres have an average size in the range from about 0.5 to 2.0 microns. With microspheres in this size range, it is preferred that the magnetic particles have diameters of not over 300 Angstroms, such as an average size of about 100 Angstroms.

The procedure previously published for preparing albumin microspheres can be used. Widder et al, J. Pharm. Sci., 68, 79 (1979). The preferred procedure is the one described for the heat-stabilized microspheres. In general, an aqueous mixture is prepared for use in forming the microcapsules, the mixture



containing the albumin or other hydrocolloid matrix polymer, Protein A, and the magnetic particles. The solid materials are dispersed in water and thoroughly mixed therewith, for example, using 20 to 40 parts of total solids per 100 parts of water. Sufficient water should be present to form an aqueous gel with the matrix hydrocolloid. In general, the amount of water may range from 10 to 60 parts per 100 parts of total solids. The aqueous mix is then emulsified with an oil, such as a vegetable oil, the emulsification being carried out with vigorous agitation, for example, using sonication, to obtain a droplet dispersion of the aqueous mix in the vegetable oil having the requisite droplet size to form the microspheres. Preferably, the emulsification is carried out at low temperatures, such as temperatures in the range of 20 to 30° C. After the emulsion has been formed, the emulsion is added to a larger body of oil, which is preferably the same oil used to form the emulsion. In practice, cottonseed oil gives good results. To promote the separation of the water droplets, the emulsion can be added in small increments to the oil bath, such as by dropwise addition. Preferably, also, the addition is accompanied by rapid stirring of the oil into which the emulsion is being introduced.


For purpose of the present invention, the droplets may be heat-hardened to stabilize them and thereby provide the microspheres. This can be conveniently accomplished by using a heated oil bath, that is, by dispersing the emulsion into hot oil, such as oil at a temperature in the range of 70 to 160°C. The effect of heat stabilization on albumin microspheres is described in United States patent 3,937,668, issued February 10, 1976.

After the heat-hardening, the prepared microspheres are separated from the oil. This may be accomplished by centrifugation.

or filtration, and the microspheres washed with a suitable organic solvent, such as diethyl ether, to remove the oil from the exterior surfaces of the microspheres. The microspheres are then ready for reaction with a specific antibody, such as an antibody prepared in rabbits. Such rabbit immunoglobulins which bind to Protein A include all subclasses of IgG. However, antibodies prepared from other sources can be used, providing they also bind to Protein A. Usually, the antibodies will be applied to the microspheres in aqueous suspension. The concentration of the antibodies may be low, since the Protein A will remove the antibodies from the treating solution even at low concentrations. As previously described, the binding is through the Fc region of the antibodies, thereby providing for an oriented attachment of the antibodies with the antigen-binding Fab arms extending outwardly from the outer surfaces of the microspheres. The microspheres are then ready for use in magnetic cell separation, as previously described in the literature.

The magnetic sorting method of this invention and the preferred microspheres for use therein are further described and illustrated in the following specific examples. For conciseness of description, the examples use certain abbreviations, which have the following meanings:

SpA: Staphylococcal Protein A
FITC: flurocein isothiocyanate
CRBC: chicken red blood cell
SRBC: sheep red blood cell
RBC: red blood cell
FCS: fetal calf serum
H2SS: Hank's balanced saline solution
EDC: carbodiimide: 1-cyclohexyl-3-(2-morpholinyl-4)-ethyl-carbodiimide methotoluene sulphonate)



EXAMPLE I


Magnetic albumin microspheres containing staphylococcal Protein A (SpA) as part of the matrix were prepared by an emulsion polymerization method. A 0.5 ml aqueous suspension containing a total of 190 mg dry material was made consisting of 66% human serum albumin, 19% Fe_3O_4 (particles 15-20 nm) and 15% SpA. To this, 60 ml of cottonseed oil was added and the emulsion was homogenized by sonication for one minute. The homogenate was added dropwise to 200 ml of constantly stirred cottonseed oil at 120 to 125°C for 10 minutes. The suspension was washed four times in diethyl ether by centrifugation for 15 minutes at 2000 xg and stored at 4°C until subsequent use. A sample of microspheres were coupled with FITC-conjugated rabbit IgG by incubation at 37°C for 20 minutes, and examined for surface fluorescence with a fluorescent microscope. The intensity and apparent uniform distribution of fluorescence indicated that SpA was oriented on the microsphere surface in a manner that allowed IgG molecules to interact with the Fc binding sites on the SpA.

EXAMPLE II

Microspheres prepared as described in Example I were used to separate CRBC from suspensions containing both CRBC and SRBC. Aliquots of CRBC and SRBC were labeled with ^{51}Cr in order to assess extent of separation as well as cell integrity. Labeling of CRBC was accomplished by incubating 1×10^8 CRBC suspended in 0.2 ml Hanks balanced salt solution (HBSS) containing 2.5% heat inactivated fetal calf serum (FCS) with $100 \mu\text{Ci Na}_2^{51}\text{CrO}_4$ (1 mCi/ml) for 90 minutes at 37°C. SRBC were labeled by similar treatment with the exception of overnight incubation at 37°C. Antibody was coupled to the

microspheres by incubating 0.5 mg of the microspheres suspended in 0.2 ml of 0.9% NaCl solution containing 0.1% Tween 80 (saline-Tween 80) with either 0.5 mg rabbit anti-chicken RBC (IgG fraction) or 0.5 mg normal rabbit IgG for 45 minutes at 37°C. Unbound IgG was removed by centrifugation with excess saline-Tween 80 at 1500 xg for two minutes at 4°C. Microspheres were then resuspended in 0.2 ml saline-Tween 80 by briefly sonicating in an ultrasonic waterbath. To this suspension, a mixture of 1×10^6 CRBC and 1×10^6 SRBC in 0.2 ml of HBSS was added. The cells were then incubated with the IgG-coated microspheres for 30 minutes at 37°C with mild agitation. Cells bearing adherent magnetic microspheres were removed from suspension by applying a 4000 gauss (gradient - 1500 gauss/cm) bar magnet to the side of each test tube for one minute. Both supernatant and pellet fractions were counted in a Beckman Model 8000 gamma counter for ^{51}Cr . Control labeled cells, incubated in saline-Tween 80, were counted for ^{51}Cr to assess spontaneous release.

Based on ^{51}Cr counts, it was found that when 1×10^6 ^{51}Cr CRBC in combination with 1×10^6 SRBC were incubated with 0.5 mg microspheres bearing anti-CRBC antibodies, 97.8% of the labeled cells were magnetically removed from suspension. Hemocytometer counts of erythrocytes in the supernatant revealed only 0.26% residual CRBC among the remaining SRBC. Using this method of cell separation, a population of SRBC which was 97-99% homogeneous was generated with 90.5% recovery of the starting SRBC mass. The non-specific adherence of ^{51}Cr CRBC was tested while using microspheres bearing anti-SRBC and normal rabbit IgG respectively, and found to be <10%.



EXAMPLE III

Microspheres prepared as described in Example I were used to fractionate Lewis rat splenocytes. Based on the presence or absence of surface immunoglobulins, it is possible to distinguish between thymus-derived T lymphocytes and bone-marrow derived B lymphocytes. Normal non-IgG bearing splenocytes, considered to be predominantly T lymphocytes, were purified by incubating splenocytes with microspheres containing rabbit anti-rat IgG.

A cellular suspension of spleen cells was obtained by teasing rat spleen on a metal screen in HBSS with 10% heat inactivated FCS. The cells were washed three times and overlaid on Ficoll-Hypaque (specific gravity 1.072). The gradient was then centrifuged at 1200 xg for 25 minutes at 25°C, to eliminate dead cells and red blood cells. The resultant interface band was removed and assessed for viability by trypan blue dye exclusion. The number of IgG bearing cells was determined by incubating the cells at 37°C with FITC conjugated rabbit anti-rat IgG and counting the number of fluorescent labeled cells. Rabbit anti-rat IgG, normal rabbit IgG, and rabbit anti-chicken RBC were coupled to 0.5 mg the SPA microspheres (0.5 mg IgG/0.5 mg microspheres). Splenocytes (2×10^6) suspended in HBSS with 2.5% heat inactivated FCS were added to the 0.5 mg microspheres. In order to minimize the capping phenomenon and maintain viability, reaction mixtures were incubated for 2.5 hours at 4°C. Cells with adherent microspheres were separated magnetically as described in Example II, and resultant supernatants were analyzed for total cell count, viability, and fluorescence. The results are summarized in Table A.

-13-

Viability of unfractionated cells after centrifugation in Ficoll-Hypaque was 96%. Supernatant cell viability after magnetic separation was 93%, demonstrating a minimal loss of viability. Between 47 to 51% of unfractionated splenocytes were IgG-bearing cells as determined by fluorescence microscopy. However, after magnetic separation of splenocytes in the experimental group, only 0.5% of the supernatant cells had detectable IgG on their surface, showing a highly enriched population of non-IgG-bearing lymphocytes.

Antibody specificity was verified by demonstrating negligible depletion of IgG-bearing cells following incubation of splenocytes with microspheres containing either normal rabbit IgG or anti-CRBC. Rat thymocytes, normally containing 4 to 6% IgG bearing lymphocytes, were totally depleted of these cells after incubation with microspheres coupled with anti-rat IgG.

The sensitivity of the system was tested by serial dilutions of microspheres bearing rabbit anti-chicken RBC with the addition of 1×10^6 ^{51}Cr CRBC at each dilution. Incubation of microspheres and cells was carried out for 30 minutes at 37°C . Cells with adherent microspheres were magnetically removed and both pellet and supernatant fractions counted for ^{51}Cr . Percent CRBC bound to the microspheres was linearly related to the amount of microspheres present until microsphere saturation occurred. No less than 99% binding of 1×10^6 CRBC was observed when $\geq 104 \mu\text{g}$ of microspheres were used.

TABLE A
Separation of Rat T and B Lymphoid Cells Using Antibodies Coupled to SpA-Bearing Magnetic Microspheres.

(Results represent two different experiments done in triplicate and expressed as mean \pm S.D.)

Antibody coupled to microspheres	Type of cell suspension (1)	% of total cells bound to microspheres	% of IgG bearing cells remaining in superna- tant after magnetic separation (2)
Rabbit anti-rat IgG	Splenocytes	50 \pm 0.5	0.5
Rabbit anti-rat IgG	Thymocytes	15 \pm 1	0 (3)
Normal rabbit IgG	Splenocytes	10 \pm 0.3	47.4 \pm 1.2 (3)
Rabbit anti-CRBC	Splenocytes	6.5 \pm 2	44 \pm 1.8 (3)

Footnotes to Table A

- (1) 2×10^6 cells/0.2 ml HBSS + 2.5% FCS, incubated with 0.5 mg microspheres bearing antibody as indicated. The reaction mixture was incubated for 2.5 hours at 4°C.
- (2) The percent of IgG-bearing cells not removed by magnetic microspheres was determined by incubating supernatant cells with 0.1 ml FITC conjugated rabbit anti-rat IgG for 20 minutes at 37°C. The amount of contaminant IgG-bearing cells was determined by fluorescence microscopy. In addition, 95.3% of the expected non-IgG-bearing splenocytes was found in the supernatant as determined by triplicate counts in a hemocytometer of non-fluorescent cells.
- (3) Between 44 to 51% starting splenocytes are IgG-bearing cells as determined by fluorescence microscopy, and 4 to 6% of rat thymocytes are IgG-bearing cells as determined by the same method.

EXAMPLE IV

Magnetic albumin microspheres were prepared as described in Example I, except that Protein A was omitted. The amount of albumin was correspondingly increased so that the dry material used to form the microspheres was 81% albumin and 19% Fe_3O_4 . Protein A can be applied to the microspheres thus formed as described in Example V.

EXAMPLE V

5mg/ml Protein A in HBSS is prepared as a starting solution. For the one step aqueous carbodiimide coupling 10 mg of EDC is added to 20 mg of either an acrylate polymer microsphere

matrix prederivatized with ϵ -aminocaproic acid, or 20 mg of albumin microspheres suspended in 10 mls of the starting Protein A solution and allowed to react for 4 hrs at 4°C with vigorous stirring. The coupling reaction is then terminated by addition of 0.4 ml of 0.2 M glycine solution pH 7.9. Unbound Protein A and unreacted EDC is removed by washing 4X in HBSS. The Protein A coated microspheres are then coupled to appropriate antisera by incubation of 2 mg of microspheres with 1 ml of antiserum at 37°C for 10 mins with slight agitation. The alternative coupling agent is glutaraldehyde 1.25% solution which is added to 10 mls of HBSS solution (pH 7.4) containing 50 mg Protein A and 20 mg of either albumin microspheres or 20 mg of diaminoheptane derivatized acrylate microspheres, allowed to react for 2 hrs at 37°C with slight agitation. Unreacted Protein A and excess glutaraldehyde is removed by centrifugation washing 4 X with HBSS. Antibody is attached to microspheres as described above in prior examples.


CLAIMS

1. A method for the separation of a select population of cells, bacteria, or viruses from a mixed population thereof, in which microspheres containing magnetic particles are coated with a layer of antibodies which selectively bind to the select population, the coated microspheres are contacted with said mixed population so that said microspheres are bound to the select population, and said bound select population is magnetically separated from the rest of said mixed population, wherein the improvement comprises: prior to coating said microspheres with antibodies modifying the surfaces of said microspheres to provide staphylococcal Protein A distributed thereover in adherent relation to said microspheres, then contacting said microspheres with antibodies which bind to Protein A and which also bind selectively to said select population, whereby said antibodies are arranged in oriented attachment on the surfaces said microspheres with their Fab arms extending outwardly, and thereafter carrying out the rest of the steps of said method.

2. The method of claim 1 in which said microspheres are formed of a polymer matrix material in admixture with said magnetic particles and said Protein A.

3. The method of claim 2 in which said matrix material is albumin and said microspheres contain from 2 to 40 parts by weight of Protein A per 100 parts of albumin.

4. The method of claim 1 in which said Protein A is chemically-bonded to the exterior surfaces of said microspheres.




5. Microspheres for magnetically sorting of cells, bacteria, or viruses, comprising microspheres formed from an amino acid polymer matrix material in admixture with staphylococcal Protein A, and being of a size from 0.2 to 100 microns diameter, said microspheres containing from 2 to 40 parts by weight of said Protein A per 100 parts of said amino acid polymer and said Protein A being present in the exterior surfaces of said microspheres for antibody binding, said microspheres also containing magnetic particles of a size from 100 to 20,000 Angstroms and in an amount sufficient to make said microspheres magnetically-responsive.

6. The microspheres of claim 5 in which said amino acid polymer is albumin.

7. The microspheres of claim 5 or claim 6 in which said Protein A is present in an amount of from 10 to 30 parts by weight per 100 parts of said amino acid polymer.

8. Microspheres for magnetically sorting of cells, bacteria, or viruses, comprising microspheres formed from an aqueous mixture of albumin, staphylococcal Protein A, and magnetic particles, said microspheres having an average diameter of from 0.5 to 2.0 microns, and containing from 2 to 40 parts of said Protein A per 100 parts of said albumin and said Protein A being present in the exterior surfaces of said microspheres for antibody binding, said magnetic particles being of a size not over 300 Angstroms and being present in an amount sufficient to make said microspheres magnetically-responsive.



-19-

9. The microspheres of claim 8 in which said magnetic particles are Fe_3O_4 and are present in an amount of from 10 to 150 parts by weight per 100 parts of said albumin.

10. The microspheres of claim 8 or claim 9 in which said Protein A is present in an amount of from 10 to 35 parts by weight per 100 parts of said albumin.

11. A method according to claim 1 in which microspheres as defined in any of claims 5 to 10, are employed.



European Patent
Office

EUROPEAN SEARCH REPORT

2016552
Application number
EP 80 30 0546

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 3)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D	<p><u>US - A - 3 970 518</u> (I. GIAEVER)</p> <p>* Whole document *</p> <p>--</p> <p>CHEMICAL ABSTRACTS, vol. 89, no. 9, 28th August 1978, page 303, column 1, no. 74026x Columbus, Ohio, U.S.A. V. GHETIE et al.: "Separation of cells by affinity chromatography on SpA-Sepharose 6MB"</p> <p>& J. IMMUNOL. METHODS 1978, 21 (1-2), 133-41</p> <p>* Abstract *</p> <p>--</p> <p>CHEMICAL ABSTRACTS, vol. 82, no. 21, 26th May 1975, page 236, column 2, no. 135217q Columbus, Ohio, U.S.A. V. GHETIE et al.: "Density gradient separation of lymphoid cells adhering to protein A-containing Staphylococci"</p> <p>& PROC. NATL. ACAD. SCI. U.S.A., 1974, 71(12), 4831-5</p> <p>* Abstract *</p> <p>--</p> <p><u>US - A - 4 092 116</u> (I. GIAEVER)</p> <p>* Column 1, lines 13-18; column 3, lines 46-49; column 4, lines 20-26; column 5, lines 24-26; claim 1 *</p> <p>--</p>	<p>1-11</p> <p>1-5,8</p> <p>1-5,8</p> <p>1-5,8</p> <p>1-5,8</p>	<p>C 12 N 1/02 7/02 C 12 Q 1/00</p> <p>TECHNICAL FIELDS SEARCHED (Int. Cl. 3)</p> <p>G 01 N 33/54 C 12 N 1/02 A 61 K 39/00 39/02 39/12 C 12 N 7/02 C 12 Q 1/00</p> <p>CATEGORY OF CITED DOCUMENTS</p> <p>X: particularly relevant A: technological background O: non-written disclosure P: intermediate document T: theory or principle underlying the invention E: conflicting application D: document cited in the application L: citation for other reasons</p> <p>&: member of the same patent family. corresponding document</p>
P	<p>BIOSIS ARTICLE BIORESEARCH INDEX 17005632 ./.</p>	1-11	
<p><input checked="" type="checkbox"/> The present search report has been drawn up for all claims</p>			
Place of search		Date of completion of the search	Examiner
The Hague		23-06-1980	GALLIGANI



European Patent
Office

EUROPEAN SEARCH REPORT

Application number
EP 80 30 0546

-2-

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. 3)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
	K.J. WIDDER et al.: "Protein A Bearing Magnetic Microspheres A New Method for Cell Sorting" & FEDERATION PROCEEDINGS, vol. 38, no. 3, Part 1, 1979, page 1285 --		
P	BIOSIS ARTICLE BIORESEARCH INDEX 18056589 K.J. WIDDER et al.: "Magnetic Protein A Microspheres a rapid method for cell separation" & CLINICAL IMMUNOLOGY AND IMMUNOPATHOLOGY, vol. 14, no. 3, 1979 pages 395-400 --	1-11	TECHNICAL FIELDS SEARCHED (Int. Cl. 3)
D	NATURE, vol. 268, no. 5619, 4th August 1977, pages 437-438 R.S. MOLDAV et al.: "Application of magnetic microspheres in labelling and separation of cells" * Whole document * --	1-11	
A	FR - A - 2 334 106 (INSTITUT PASTEUR) * Claims 1-3 * ----	1-11	

(18)



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

0 016 552
B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: **13.10.82**

(51) Int. Cl.³: **C 12 N 1/02, C 12 N 7/02,**
C 12 Q 1/00

(21) Application number: **80300546.1**

(22) Date of filing: **25.02.80**

(54) Method of magnetic separation of cells and the like, and microspheres for use therein.

(30) Priority: **28.02.79 US 15895**

(43) Date of publication of application:
01.10.80 Bulletin 80/20

(45) Publication of the grant of the patent:
13.10.82 Bulletin 82/41

(84) Designated Contracting States:
AT BE CH DE FR GB IT LU NL SE

(56) References cited:
FR - A - 2 334 106
US - A - 3 970 518
US - A - 4 092 116

CHEMICAL ABSTRACTS, vol. 89, no. 9, 28th
August 1978, page 303, column 1, no. 74026x
Columbus, Ohio, U.S.A. V. GHETIE et al.:
"Separation of cells by affinity chromatography
on SpA-Sepharose 6MB"
CHEMICAL ABSTRACTS, vol. 82, no. 21, 26th
May 1975, page 236, column 2, no. 135217q
Columbus, Ohio, U.S.A. V. GEHTIE et al.:
"Density gradient separation of lymphoid cells
adhering to protein A-containing Staphylococci"

(73) Proprietor: **Kendrew Biosystems, Inc.**
11180 Roselle Street
San Diego, Cal. (US)

(72) Inventor: **Widder, Kenneth, Jon**
115, Carol Street
Carrboro North Carolina 27510 (US)
Inventor: **Senyei, Andrew Erwin**
14-1/2 38th Place
Long Beach California 90803, (US)

(74) Representative: **Patentanwälte Grünecker, Dr.**
Kinkeldey, Dr. Stockmair, Dr. Schumann, Jakob,
Dr. Bezold, Meister, Hilgers, Dr. Meyer-Plath
Maximilianstrasse 43
D-8000 München 22 (DE)

(56) References cited:
BIOSIS ARTICLE BIORESEARCH INDEX
17005632 K. J. WIDDER et al.: "Protein A
Bearing Magnetic Microspheres A New Method
for Cell Sorting"
BIOSIS ARTICLE BIORESEARCH INDEX
18056589 K. J. WIDDER et al.: "Magnetic
Protein A Microspheres a rapid method for cell
separation"

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European patent convention).

Courier Press, Leamington Spa, England.

EP 0 016 552 B1

**NATURE, vol. 268, no. 5619, 4th August 1977,
pages 437—438 R. S. MOLDAY et al.:
"Application of magnetic microspheres in
labelling and separation of cells" Whole
document**

Method of magnetic separation of cells and the like, and microspheres for use therein

This invention relates to the fractionation of heterogeneous populations of cells or the like to isolate a relatively homogeneous sub-population of a specific cell type. More specifically, the improvement of this invention relates to magnetic sorting of cells, bacteria, or viruses.

A general procedure for magnetic sorting of cells, bacteria, and viruses is disclosed in US—A—3,970,518. In that procedure, uncoated particles of a magnetic material, such as iron oxide, are contacted with a high concentration liquid dispersion of the selective antibody, and after sufficient antibody has adhered to the magnetic particles, the coated particles are contacted with the mixed population to be fractionated, the select cell or the like binding to the magnetic particles, and the bound cells are then separated magnetically from the remainder of the population. As a further step, the select cells may be separated from the magnetic material, by the use of a cleaving agent solution and magnetic removal of the magnetic particles.

While there are literature reports describing the use of magnetic microspheres in cell sorting, there is no literature verification that uncoated magnetic particles can be made to effectively bind with antibodies. In the published procedures, the particles of magnetic material are contained in microspheres formed from polymers, which can be chemically coupled to antibodies. See, for example: Molday et al, *Nature*, 268, 437 (1977); Kronick et al, *Science*, 200, 1074 (1978); and Antoine et al, *Immunochemistry*, 15, 443 (1978). These references describe magnetically-responsive microspheres formed from acrylate polymers, such as hydroxyethyl methacrylate, or polyacrylamide-agarose microspheres. Such microspheres can be chemically coupled to antibodies with glutaraldehyde or other dialdehyde. As described by the cited Molday (1977) and Kornick references, one procedure involves the chemical attachment of diaminoheptane spacer groups to the microspheres, which are then chemically linked to the antibodies by glutaraldehyde reaction. Although effective bonding of the antibodies can be obtained, such procedures are difficult since aggregation of microspheres can readily occur and the preparative procedure is time consuming. For example, the reaction to attach spacer groups may require from five to twelve hours of chemical reaction time, and subsequent dialysis to remove the excess reagent. The coupling of the antibodies may then require another twelve to twenty-four hours followed by dialysis to remove excess coupling agent. Further, such antibody reagents may not be used efficiently, since an excess of the antibodies will usually need to be present during the chemical coupling.

Another disadvantage of magnetic particle or microsphere separation methods as described in the art is that the antibodies are attached to the microspheres in a random manner. Antigen-binding occurs through the Fab regions of the antibodies which are in the outer portions of the arms. With random attachment of the antibodies, one or both of the Fab arms may be unavailable for antigen-binding. Thus, an excess of antibody must be used to assure that the coated microspheres effectively bind to the antigens associated with the cells or other bodies being sorted.

The present invention utilizes staphylococcal Protein A to overcome the limitations of prior art magnetic sorting procedures, as described above. It is known that staphylococcal Protein A selectively binds to antibodies through the Fc region of the antibodies, which is located in the tail portions of the antibodies remote from the Fab arms. See Forsgren et al, *J. Immunol.*, 99, 19 (1967). Heretofore, however, this property of Protein A has not been utilized to form magnetic microspheres. Protein A has been coupled to Sepharose beads (cross-linked agarose gels) to provide a column material with immunoglobulin-binding properties. The column may be used for affinity chromatography, for example, of the IgG fraction of serum. Such chromatographic column materials are commercially available.

Protein A has also been used in procedures for cell separation by density gradient centrifugation. See, for example, Ghetie et al, *Scand. J. Immunol.*, 4, 471 (1975). In a typical procedure, sheep erythrocytes are coated with Protein A by CrCl_3 coupling, and the coated erythrocytes are then contacted with mouse lymphocytes which have been previously reacted with antibodies to prepare the cell surfaces for binding to Protein A, thereby resulting in rosetting of the lymphocytes around the erythrocytes. The resulting rosetted cells are recovered by density gradient centrifugation.

In accordance with the present invention, as distinguished from prior art procedures, magnetically-responsive microspheres are prepared having Protein A associated with the surfaces thereof, and the resulting microspheres are first reacted with the select antibodies before the microspheres are used for cell separation. With the microspheres used in the method of this invention the antibodies are thereby arranged in oriented attachment on their outer surfaces with the Fab arms of the antibodies extending outwardly. The effectiveness of the microspheres for antigen binding and use in magnetic sorting procedures is thereby maximized. This greatly increases the efficiency with which the select antibodies may be used. Further, it eliminates the need for chemical coupling of the antibodies.

In a preferred embodiment, the microspheres are prepared by mixing Protein A with a polymer matrix material which does not mask the antibody-binding sites of the Protein A. The resulting microspheres having the Protein A in the outer surfaces thereof do not require chemical coupling of the Protein A to preformed microspheres. Albumin appears to be a particularly suitable matrix material for preparing such microspheres. When the microspheres are formed from an aqueous admixture of albumin, Protein A, and magnetic particles, the Protein A is effectively available in the outer surfaces of the microspheres, in effect, forming surface layers on the microspheres with the Protein A in high concentration. The explanation for this result is not fully understood, but appears to relate to the wetting agent or surface tension properties of Protein A when dispersed in an aqueous solution in admixture with albumin.

Thus the present invention comprises a method for separation of a select population of cells, bacteria, or viruses from a mixed population thereof, in which the microspheres containing magnetic particles are coated with a layer of antibodies which selectively bind to the select population. The coated microspheres are contacted with the mixed population, and the bound select population is magnetically separated from the rest of the mixed population. The method improvement is characterized by modifying the surfaces of the microspheres prior to coating them with antibodies to provide staphylococcal Protein A distributed thereover in adherent relation to the microspheres. The microspheres are then contacted with antibodies which bind the Protein A and which also bind selectively to the select population. By this means the antibodies are arranged in oriented attachment on the surfaces of the microspheres with their Fab arms extending outwardly. Thereafter, the rest of the steps of the magnetic separation are carried out, as is known in the art. Preferably, the microspheres are formed of a polymer matrix material in admixture with the magnetic particles and Protein A, such as an albumin matrix material in an amount of 100 parts per 5 to 40 parts of Protein A. Alternatively, however, the Protein A may be chemically-bonded to the exterior surfaces of the microspheres to provide a Protein A coating thereon.

Where chemical coupling procedures are used, the microspheres may be formed from any matrix material which can be chemically coupled to Protein A, including albumin or other amino acid polymer, and synthetic polymers, such as acrylate polymers. For example, the microspheres may be formed from methyl methacrylate, hydroxyethyl methacrylate, methacrylic acid, ethylene glycol dimethacrylate, agarose polymers, polyacrylamide polymers, or mixtures of such polymers. Protein A may be directly coupled to solid support

surfaces containing magnetically responsive materials by several procedures. See, for example, Molday et al, *J. Cell Biology*, 64, 75 (1975) Microspheres can be derivatized with either aminocaproic acid or diaminoheptane which provide extend functional groups for coupling proteins to insolubilized matrixes. Alternatively, with solid surfaces already containing functionally available groups (i.e. amino groups on albumin microspheres) a direct glutaraldehyde coupling of Protein A may be accomplished.

An alternate preferred procedure is to incorporate the Protein A in the microspheres by admixing it with the matrix material prior to the formation of the microspheres, and carrying out the preparation so that the Protein A is available in the outer surfaces of the microspheres. Suitable procedures for preparing such microspheres will therefore be described, but it should be understood that the present invention in its broad method aspect is not limited to the use of such preferred microspheres.

For use in the present invention, the Protein A can be prepared from *Staphylococcus aureus* by procedures described in the literature. See, for example, Forsgren et al, *J. Immun.*, 97, 822 (1966); and Konvall et al, *Immunochemistry*, 7, 124 (1970). Staphylococcal Protein A is also available from commercial sources, such as Pharmacia Fine Chemicals, Piscataway, New Jersey.

The preferred matrix material for forming the microspheres by admixture with Protein A is an amino acid polymer, such as albumin. Animal or human albumin may be used, for example, human serum albumin. Other water-soluble proteins can be used such as hemoglobin, or synthetic amino acid polymers including poly-L-lysine and poly-L-glutamic acid.

When the Protein A is premixed with the matrix polymer and the microspheres formed therefrom, sufficient Protein A should be included so that the outer surfaces of the microspheres will bind antibodies through the selective action of the Protein A. In general, the microspheres may contain from 2 to 40 parts by weight of Protein A per 100 parts of the matrix polymer such as albumin. Preferred proportions are from about 10 to 35 parts of the Protein A per 100 parts of the matrix polymer.

A sufficient amount of finely-divided particles of a magnetic material should also be included so that the microspheres are magnetically-responsive. For example, the magnetic particles may be ferri- or ferro-magnetic compounds, such as magnetic iron oxides. Other useable magnetic materials in particulate form are disclosed in U.S.—A—3,970,518. A preferred magnetic material is magnetite (Fe_3O_4). Depending on the size of the microspheres, the magnetic particles may range in size from 10 to 2000 nm. The microspheres may contain from 10 to 150 parts by weight of the magnetic material per 100 parts of the matrix polymer.

The microspheres may range in size from 0.2 to 100 μm in diameter. Preferably, however, the microspheres have an average size in the range from about 0.5 to 2.0 μm . With microspheres in this size range, it is preferred that the magnetic particles have diameters of not over 30 nm, such as an average size of about 10 nm.

The procedure previously published for preparing albumin microspheres can be used. Widder et al, *J. Pharm. Sci.*, 68, 79 (1979). The preferred procedure is the one described for the heat-stabilized microspheres. In general, an aqueous mixture is prepared for use in forming the microcapsules, the mixture containing the albumin or other hydrocolloid matrix polymer, Protein A, and the magnetic particles. The solid materials are dispersed in water and thoroughly mixed therewith, for example, using 20 to 40 parts of total solids per 100 parts of water. Sufficient water should be present to form an aqueous gel with the matrix hydrocolloid. In general, the amount of water may range from 10 to 60 parts per 100 parts of total solids. The aqueous mix is then emulsified with an oil, such as a vegetable oil, the emulsification being carried out with vigorous agitation, for example, using sonication, to obtain a droplet dispersion of the aqueous mix in the vegetable oil having the requisite droplet size to form the microspheres. Preferably, the emulsification is carried out at low temperatures, such as temperatures in the range of 20 to 30°C. After the emulsion has been formed, the emulsion is added to a larger body of oil, which is preferably the same oil used to form the emulsion. In practice, cottonseed oil gives good results. To promote the separation of the water droplets, the emulsion can be added in small increments to the oil bath, such as by dropwise addition. Preferably, also, the addition is accompanied by rapid stirring of the oil into which the emulsion is being introduced.

For purpose of the present invention, the droplets may be heat-hardened to stabilize them and thereby provide the microspheres. This can be conveniently accomplished by using a heated oil bath, that is, by dispersing the emulsion into hot oil, such as oil at a temperature in the range of 70 to 160°C. The effect of heat stabilization on albumin microspheres is described in US—A—3,937,668.

After the heat-hardening, the prepared microspheres are separated from the oil. This may be accomplished by centrifugation or filtration, and the microspheres washed with a suitable organic solvent, such as diethyl ether, to remove the oil from the exterior surfaces of the microspheres. The microspheres are then ready for reaction with a specific antibody, such as an antibody prepared in rabbits. Such rabbit immunoglobulins which bind to Protein A include all subclasses of IgG. However, antibodies prepared from other sources can be used, providing they also bind to Protein A. Usually, the antibodies will be applied to the

microspheres in aqueous suspension. The concentration of the antibodies may be low, since the Protein A will remove the antibodies from the treating solution even at low concentrations. As previously described, the binding is through the Fc region of the antibodies, thereby providing for an oriented attachment of the antibodies with the antigen-binding Fab arms extending outwardly from the outer surfaces of the microspheres. The microspheres are then ready for use in magnetic cell separation, as previously described in the literature.

The magnetic sorting method of this invention and the preferred microspheres for use therein are further described and illustrated in the following specific examples. For conciseness of description, the examples use certain abbreviations, which have the following meanings:

SpA: Staphylococcal Protein A
 FITC: fluorecein isothiocyanate
 CRBC: chicken red blood cell
 SRBC: sheep red blood cell
 RBC: red blood cell
 FCS: fetal calf serum
 HBSS: Hank's balanced saline solution
 EDC: carbodiimide: 1-cyclohexyl-3-(2-morpholinyl-4)-ethyl-carbodiimide
 methotoluene sulphonate)

Example I

Magnetic albumin microspheres containing staphylococcal Protein A (SpA) as part of the matrix were prepared by an emulsion polymerization method. A 0.5 ml aqueous suspension containing a total of 190 mg dry material was made consisting of 66% human serum albumin, 19% Fe_3O_4 (particles 15—20 nm) and 15% SpA. To this, 60 ml of cottonseed oil was added and the emulsion was homogenized by sonication for one minute. The homogenate was added dropwise to 200 ml of constantly stirred cottonseed oil at 120 to 125°C for 10 minutes. The suspension was washed four times in diethyl ether by centrifugation for 15 minutes at 2000 xg and stored at 4°C until subsequent use. A sample of microspheres were coupled with FITC-conjugated rabbit IgG by incubation at 37°C for 20 minutes, and examined for surface fluorescence with a fluorescent microscope. The intensity and apparent uniform distribution of fluorescence indicated that SpA was oriented on the microsphere surface in a manner that allowed IgG molecules to interact with the Fc binding sites on the SpA.

Example II

Microspheres prepared as described in Example I were used to separate CRBC from suspensions containing both CRBC and SRBC. Aliquots of CRBC and SRBC were labeled with ^{51}Cr in order to assess extent of separation as well as cell integrity. Labeling of CRBC was accomplished by incubating 1×10^8 CRBC

suspended in 0.2 ml Hanks balanced salt solution (HBSS) containing 2.5% heat inactivated fetal calf serum (FCS) with 100 μ Ci $\text{Na}_2^{51}\text{CrO}_4$ (1 mCi/ml) for 90 minutes at 37°C. SRBC were labeled by similar treatment with the exception of overnight incubation at 37°C. Antibody was coupled to the microspheres by incubating 0.5 mg of the microspheres suspended in 0.2 ml of 0.9% NaCl solution containing 0.1% Tween 80 (saline-Tween 80) with either 0.5 mg rabbit anti-chicken RBC (IgG fraction) or 0.5 mg normal rabbit IgG for 45 minutes at 37°C. Unbound IgG was removed by centrifugation with excess saline-Tween 80 at 1500 xg for two minutes at 4°C. Microspheres were then resuspended in 0.2 ml saline-Tween 80 by briefly sonicating in an ultrasonic water-bath. To this suspension, a mixture of 1×10^6 CRBC and 1×10^6 SRBC in 0.2 ml of HBSS was added. The cells were then incubated with the IgG-coated microspheres for 30 minutes at 37°C with mild agitation. Cells bearing adherent magnetic microspheres were removed from suspension by applying a 4000 gauss (gradient—1500 gauss/cm) bar magnet to the side of each test tube for one minute. Both supernatant and pellet fractions were counted in a Beckman Model 8000 gamma counter for ^{51}Cr . Control labeled cells, incubated in saline-Tween 80, were counted for ^{51}Cr to assess spontaneous release.

Based on ^{51}Cr counts, it was found that when 1×10^6 ^{51}Cr CRBC in combination with 1×10^6 SRBC were incubated with 0.5 mg microspheres bearing anti-CRBC antibodies, 97.8% of the labeled cells were magnetically removed from suspension. Hemocytometer counts of erythrocytes in the supernatant revealed only 0.26% residual CRBC among the remaining SRBC. Using this method of cell separation, a population of SRBC which was 97–99% homogeneous was generated with 90.5% recovery of the starting SRBC mass. The non-specific adherence of ^{51}Cr CRBC was tested while using microspheres bearing anti-SRBC and normal rabbit IgG respectively, and found to be <10%.

Example III

Microspheres prepared as described in Example I were used to fractionate Lewis rat splenocytes. Based on the presence or absence of surface immunoglobulins, it is possible to distinguish between thymus-derived T lymphocytes and bone-marrow derived B lymphocytes. Normal non-IgG bearing splenocytes, considered to be predominantly T lymphocytes, were purified by incubating splenocytes with microspheres containing rabbit anti-rat IgG.

A cellular suspension of spleen cells was

obtained by teasing rat spleen on a metal screen in HBSS with 10% heat inactivated FCS. The cells were washed three times and over-layered on Ficoll-Hypaque (specific gravity 1.072). The gradient was then centrifuged at 1200 xg for 25 minutes at 25°C, to eliminate dead cells and red blood cells. The resultant interface band was removed and assessed for viability by trypan blue dye exclusion. The number of IgG bearing cells was determined by incubating the cells at 37°C with FITC conjugated rabbit anti-rat IgG and counting the number of fluorescent labeled cells. Rabbit anti-rat IgG, normal rabbit IgG, and rabbit anti-chicken RBC were coupled to 0.5 mg the SpA microspheres (0.5 mg IgG/0.5 mg microspheres). Splenocytes (2×10^6) suspended in HBSS with 2.5% heat inactivated FCS were added to the 0.5 mg microspheres. In order to minimize the capping phenomenon and maintain viability, reaction mixtures were incubated for 2.5 hours at 4°C. Cells with adherent microspheres were separated magnetically as described in Example II, and resultant supernatants were analyzed for total cell count, viability, and fluorescence. The results are summarized in Table A.

Viability of unfractionated cells after centrifugation in Ficoll-Hypaque was 96%. Supernatant cell viability after magnetic separation was 93%, demonstrating a minimal loss of viability. Between 47 to 51% of unfractionated splenocytes were IgG-bearing cells as determined by fluorescence microscopy. However, after magnetic separation of splenocytes in the experimental group, only 0.5% of the supernatant cells had detectable IgG on their surface, showing a highly enriched population of non-IgG-bearing lymphocytes.

Antibody specificity was verified by demonstrating negligible depletion of IgG-bearing cells following incubation of splenocytes with microspheres containing either normal rabbit IgG or anti-CRBC. Rat thymocytes, normally containing 4 to 6% IgG bearing lymphocytes, were totally depleted of these cells after incubation with microspheres coupled with anti-rat IgG.

The sensitivity of the system was tested by serial dilutions of microspheres bearing rabbit anti-chicken RBC with the addition of 1×10^6 ^{51}Cr CRBC at each dilution. Incubation of microspheres and cells was carried out for 30 minutes at 37°C. Cells with adherent microspheres were magnetically removed and both pellet and supernatant fractions counted for ^{51}Cr . Percent CRBC bound to the microspheres was linearly related to the amount of microspheres present until microsphere saturation occurred. No less than 99% binding of 1×10^6 CRBC was observed when $\geq 104 \mu\text{g}$ of microspheres were used.

TABLE A
Separation of rat T and B lymphoid cells using antibodies coupled to
SpA-bearing magnetic microspheres
(Results represent two different experiments done in triplicate
and expressed as mean \pm S.D.)

Antibody coupled to microspheres	Type of cell suspension ⁽¹⁾	% of total cells bound to microspheres	% of IgG bearing cells remaining in supernatant after magnetic separation ⁽²⁾
Rabbit anti-rat IgG	Splenocytes	50 \pm 0.5	0.5
Rabbit anti-rat IgG	Thymocytes	15 \pm 1	0
Normal rabbit IgG	Splenocytes	10 \pm 0.3	47.4 \pm 1.2 ⁽³⁾
Rabbit anti-CRBC	Splenocytes	6.5 \pm 2	44 \pm 1.8 ⁽³⁾

Footnotes to Table A

- (1) 2×10^6 cells/0.2 ml HBSS+2.5% FCS, incubated with 0.5 mg microspheres bearing antibody as indicated. The reaction mixture was incubated for 2.5 hours at 4°C.
- (2) The percent of IgG-bearing cells not removed by magnetic microspheres was determined by incubating supernatant cells with 0.1 ml FITC conjugated rabbit anti-rat IgG for 20 minutes at 37°C. The amount of contaminant IgG-bearing cells was determined by fluorescence microscopy. In addition, 95.3% of the expected non-IgG-bearing splenocytes was found in the supernatant as determined by triplicate counts in a hemocytometer of non-fluorescent cells.
- (3) Between 44 to 51% starting splenocytes are IgG-bearing cells as determined by fluorescence microscopy, and 4 to 6% of rat thymocytes are IgG-bearing cells as determined by the same method.

Example IV

Magnetic albumin microspheres were prepared as described in Example I, except that Protein A was omitted. The amount of albumin was correspondingly increased so that the dry material used to form the microspheres was 81% albumin and 19% Fe₃O₄. Protein A can be applied to the microspheres thus formed as described in Example V.

Example V

5 mg/ml Protein A in HBSS is prepared as a starting solution. For the one step aqueous carbodiimide coupling 10 mg of EDC is added to 20 mg of either an acrylate polymer microsphere matrix prederivatized with ϵ -aminocaproic acid, or 20 mg of albumin microspheres suspended in 10 mls of the starting Protein A solution and allowed to react for 4 hrs at 4°C with vigorous stirring. The coupling reaction is then terminated by addition of 0.4 ml of 0.2 M glycine solution pH 7.9. Unbound Protein A and unreacted EDC is removed by washing 4x in HBSS. The Protein A coated microspheres are then coupled to appropriate antisera by incubation of 2 mg of microspheres with 1 ml of antiserum at 37°C for 10 mins with slight agitation. The alternative coupling agent is glutaraldehyde 1.25% solution which is added to 10 mls of HBSS solution (pH 7.4) containing 50 mg Protein A and 20 mg of either albumin microspheres or 20 mg of diaminoheptane derivatized acrylate microspheres, allowed to react for 2 hrs at 37°C with slight agitation. Unreacted Protein A and excess glutaraldehyde is removed by centrifugation washing 4x with HBSS. Antibody is attached to

microspheres as described above in prior examples.

Claims

1. A method for the separation of a select population of cells, bacteria, or viruses from a mixed population thereof, in which microspheres containing magnetic particles are coated with a layer of antibodies which selectively bind to the select population, the coated microspheres are contacted with said mixed population so that said microspheres are bound to the select population, and said bound select population is magnetically separated from the rest of said mixed population, characterized in that prior to coating said microspheres with antibodies, modifying the surfaces of said microspheres to provide staphylococcal Protein A distributed thereover in adherent relation to said microspheres, then contacting said microspheres with antibodies which bind to Protein A and which also bind selectively to said select population, whereby said antibodies are arranged in oriented attachment on the surfaces said microspheres with their Fab arms extending outwardly.

2. The method of claim 1 in which said microspheres are formed of a polymer matrix material in admixture with said magnetic particles and said Protein A.

3. The method of claim 2 in which said matrix material is albumin and said microspheres contain from 2 to 40 parts by weight of Protein A per 100 parts of albumin.

4. The method of claim 1 in which said

Protein A is chemically-bonded to the exterior surfaces of said microspheres.

5. Microspheres for magnetically sorting of cells, bacteria, or viruses, comprising microspheres formed from an amino acid polymer matrix material in admixture with staphylococcal Protein A, and being of a size from 0.2 to 100 μm diameter, said microspheres containing from 2 to 40 parts by weight of said Protein A per 100 parts of said amino acid polymer and said Protein A being present in the exterior surfaces of said microspheres for antibody binding, said microspheres also containing magnetic particles of a size from 10 to 2000 nm and in an amount sufficient to make said microspheres magnetically-responsive.

6. The microspheres of claim 5 in which said amino acid polymer is albumin.

7. The microspheres of claim 5 or claim 6 in which said Protein A is present in an amount of from 10 to 30 parts by weight per 100 parts of said amino acid polymer.

8. Microspheres for magnetically sorting of cells, bacteria, or viruses, comprising microspheres formed from an aqueous mixture of albumin, staphylococcal Protein A, and magnetic particles, said microspheres having an average diameter of from 0.5 to 2.0 μm , and containing from 2 to 40 parts of said Protein A per 100 parts of said albumin and said Protein A being present in the exterior surfaces of said microspheres for antibody binding, said magnetic particles being of a size not over 30 nm and being present in an amount sufficient to make said microspheres magnetically-responsive.

9. The microspheres of claim 8 in which said magnetic particles are Fe_3O_4 and are present in an amount of from 10 to 150 parts by weight per 100 parts of said albumin.

10. The microspheres of claim 8 or claim 9 in which said Protein A is present in an amount of from 10 to 35 parts by weight per 100 parts of said albumin.

11. A method according to claim 1 in which microspheres as defined in any of claims 5 to 10, are employed.

Revendications

1. Un procédé pour la séparation d'une population choisie de cellules, bactéries ou virus d'une population mélangée de tels corps suivant lequel des microsphères contenant des particules magnétiques sont enrobées d'une couche d'anticorps qui se lient sélectivement à la population choisie, les microsphères enrobées sont mises en contact avec ladite population mélangée et ladite population choisie liée est séparée magnétiquement du reste de ladite population, caractérisé en ce qu'avant de revêtir lesdites microsphères d'anticorps, on modifie les surfaces desdites microsphères pour fournir de la Protéine A staphylococcique répartie sur elles dans une relation

d'adhérence auxdites microsphères puis on met en contact lesdites microsphères avec des anticorps qui se lient à la Protéine A et qui se lient également sélectivement à ladite population choisie, de telle sorte que lesdits anticorps sont disposés dans une fixation orientée sur les surfaces desdites microsphères avec leurs bras Fab s'étendant vers l'extérieur.

2. Procédé de la revendication 1 dans lequel lesdites microsphères sont formées en une matière de matrice constituée par un polymère en mélange avec lesdites particules magnétiques et ladite Protéine A.

3. Procédé de la revendication 2 dans lequel ladite matière de matrice est de l'albumine et lesdites microsphères contiennent de 2 à 40 parties en poids de Protéine A pour 100 parties d'albumine.

4. Procédé de la revendication 1 dans lequel la Protéine A est chimiquement liée aux surfaces extérieures desdites microsphères.

5. Microsphères pour tirer magnétiquement des cellules, bactéries et virus comprenant des microsphères formées en une matière de matrice qui est un polymère d'acide aminé en mélange avec de la Protéine A staphylococcique et qui ont une taille comprise entre 0,2 et 100 μm de diamètre, lesdites microsphères contenant de 2 à 40 parties en poids de ladite Protéine A pour 100 parties dudit polymère d'acide aminé et ladite protéine A étant présente dans les surfaces extérieures desdites microsphères pour la fixation d'anticorps, lesdites microsphères contenant également des particules magnétiques ayant une taille comprise entre 10 et 2000 nm et en quantité suffisante pour rendre lesdites microsphères magnétiquement sensibles.

6. Les microsphères de la revendication 5 dans lesquelles le polymère d'acide aminé est de l'albumine.

7. Les microsphères de la revendication 5 ou de la revendication 6 dans lesquelles ladite Protéine A est présente dans une proportion comprise entre 10 et 30 parties en poids pour 100 parties dudit polymère d'acide aminé.

8. Microsphères pour le triage magnétique de cellules, bactéries ou virus comprenant des microsphères formées à partir d'un mélange aqueux d'albumine, de Protéine A staphylococcique et de particules magnétiques, lesdites microsphères ayant un diamètre moyen compris entre 0,5 et 2,0 μm et contenant de 2 à 40 parties de ladite Protéine A pour 100 parties de ladite albumine et ladite Protéine A étant présente dans les surfaces extérieures desdites microsphères pour la fixation d'anticorps, lesdites particules magnétiques ayant une taille inférieure à 30 nm et étant présentes en une quantité suffisante pour rendre lesdites microsphères magnétiquement sensibles.

9. Les microsphères de la revendication 8 dans lesquelles lesdites particules magnétiques sont du Fe_3O_4 et sont présentes dans une

proportion comprise entre 10 à 150 parties en poids pour 100 parties de ladite albumine.

10. Les microsphères de la revendication 8 ou de la revendication 9 dans lesquelles ladite Protéine A est présente dans une proportion comprise entre 10 et 35 parties en poids de ladite albumine.

11. Procédé selon la revendication 1 dans lequel des microsphères telles que définies dans l'une quelconque des revendications 5 à 10 sont utilisées.

Patentansprüche

1. Methode zum Abtrennen einer ausgewählten Population von Zellen, Bakterien oder Viren aus einer gemischten Population davon, beider Mikrosphären, die magnetische Teilchen enthalten, mit einer Schicht von Antikörpern überzogen werden, die selektiv an die ausgewählte Population binden, wobei die überzogenen Mikrosphären mit der gemischten Population in Kontakt gebracht werden, so daß die Mikrosphären an die ausgewählte Population gebunden werden und die gebundene ausgewählte Population magnetisch von dem Rest der gemischten Population abgetrennt wird, dadurch gekennzeichnet, daß vor dem Überziehen der Mikrosphären mit Antikörpern, die Oberfläche der Mikrosphären modifiziert wird zur Bereitstellung eines von Staphylococcen stammenden Protein A, das darüber verteilt ist in haftender Beziehung an den Mikrosphären, worauf die Mikrosphären mit Antikörpern in Kontakt gebracht werden, die an Protein A binden und die auch selektiv an die gewählte Population binden, wodurch die Antikörper in orientierter Befestigung an den Oberflächen der Mikrosphären angeordnet werden, wobei ihre Fab-Arme sich nach auswärts erstrecken.

2. Methode nach Anspruch 1, bei der die Mikrosphären aus einem Polymermatrixmaterial im Gemisch mit den magnetischen Teilchen und dem Protein A gebildet werden.

3. Methode nach Anspruch 2, in der das Matrixmaterial Albumin ist und die Mikrosphären 2 bis 40 Gew.-Teile Protein A pro 100 Teile Albumin enthalten.

4. Methode nach Anspruch 1, in der das Protein A chemisch an die äußeren Ober-

flächen der Mikrosphären gebunden ist.

5. Mikrosphären zum magnetischen Sortieren von Zellen, Bakterien oder Viren, enthaltend Mikrosphären, gebildet aus einem Aminosäurepolymermatrixmaterial, im Gemisch mit von Staphylococcen stammendem Protein A und mit einer Größe von 0,2 bis 100 µm Durchmesser, wobei die Mikrosphären 2 bis 40 Gew.-Teile des Protein A pro 100 Teile des Aminosäurepolymeren enthalten und das Protein A in den äußeren Oberflächen der Mikrosphären zur Bindung von Antikörpern vorhanden ist, wobei die Mikrosphären auch magnetische Teilchen mit einer Größe von 10 bis 2000 nm und in ausreichender Menge enthalten, um die Mikrosphären magnetisch reagierend zu machen.

6. Mikrosphären nach Anspruch 5, in denen das Aminosäurepolymere Albumin ist.

7. Mikrosphären nach Anspruch 5 oder Anspruch 6, in denen das Protein A in einer Menge von 10 bis 30 Gew.-Teilen pro 100 Teile des Aminosäurepolymeren vorhanden ist.

8. Mikrosphären zum magnetischen Sortieren von Zellen, Bakterien oder Viren, enthaltend Mikrosphären, gebildet aus einem wäßrigen Gemisch von Albumin, von Staphylococcen stammendem Protein A und magnetischen Teilchen, wobei die Mikrosphären einen durchschnittlichen Durchmesser von 0,5 bis 2,0 µm aufweisen und 2 bis 40 Teile des Protein A pro 100 Teile des Albumins enthalten und das Protein A in den äußeren Oberflächen der Mikrosphären zur Antikörperbindung vorhanden ist, wobei die magnetischen Teilchen eine Größe von nicht über 30 nm aufweisen und in ausreichender Menge vorhanden sind, um die Mikrosphären magnetisch reagierend zu machen.

9. Mikrosphären nach Anspruch 8, in denen die magnetischen Teilchen Fe_3O_4 sind und in einer Menge von 10 bis 150 Gew.-Teilen pro 100 Teile des Albumins vorhanden sind.

10. Mikrosphären nach Anspruch 8 oder Anspruch 9, in denen das Protein A in einer Menge von 10 bis 35 Gew.-Teilen pro 100 Teile des Albumins vorhanden sind.

11. Methode nach Anspruch 1, bei der die Mikrosphären, wie in einem der Ansprüche 5 bis 10 definiert, verwendet werden.

55

60

65



Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number:

**0 016 552
B2**

(12)

NEW EUROPEAN PATENT SPECIFICATION

(46) Date of publication of the new patent specification:
13.01.83

(21) Application number: **80300548.1**

(22) Date of filing: **25.02.80**

(51) Int. Cl.: **C 12 N 1/02, C 12 N 7/02,
C 12 Q 1/00**

(54) **Method of magnetic separation of cells and the like, and microspheres for use therein.**

(30) Priority: **28.02.79 US 15895**

(43) Date of publication of application:
01.10.80 Bulletin 80/20

(45) Publication of the grant of the patent:
13.10.82 Bulletin 82/41

(45) Mention of the opposition decision:
13.01.88 Bulletin 88/2

(84) Designated Contracting States:
AT BE CH DE FR GB IT LU NL SE

(56) References cited:
**DE-A-2 654 723
FR-A-2 334 106
US-A-3 970 518
US-A-3 970 518
US-A-4 092 116**

CHEMICAL ABSTRACTS, vol. 89, no. 9, 28th August 1978, page 303, column 1, no. 74026x Columbus, Ohio, U.S.A. V. GHETIE et al.: "Separation of cells by affinity chromatography on SpA-Sepharese 6MB"
CHEMICAL ABSTRACTS, vol. 82, no. 21, 26th May 1975, page 236, column 2, no. 135217q Columbus, Ohio, U.S.A. V. GEHTIE et al.: "Density gradient separation of lymphoid cells adhering to protein A-containing Staphylococci"
BIOSIS ARTICLE BIORESEARCH INDEX 17005632 K.J. WIDDER et al.: "Protein A Bearing Magnetic Microspheres A New Method for Cell Sorting"

(73) Proprietor: **Kendrew Biosystems, Inc., 11180 Roselle Street, San Diego, Cal. (US)**

(72) Inventor: **Widder, Kenneth, Jon, 115, Carol Street, Carrboro North Carolina 27510 (US)**
Inventor: **Senyel, Andrew Erwin, 14- 1/2 38th Place, Long Beach California 90803, (US)**

(74) Representative: **Patentanwälte Grünecker, Dr. Kinkeldey, Dr. Stockmair, Dr. Schumann, Jakob, Dr. Bezold, Meister, Hilgers, Dr. Meyer-Plath, Maximilianstrasse 58, D-8000 München 22 (DE)**

(56) References cited: (continuation)
BIOSIS ARTICLE BIORESEARCH INDEX 18066589 K.J. WIDDER et al.: "Magnetic Protein A Microspheres a rapid method for cell separation" NATURE, vol. 268, no. 5619, 4th August 1977, pages 437-438 R.S. MOLDAY et al.: "Application of magnetic microspheres in labelling and separation of cells" Whole document J. Immunology 99 No. 1 (1967), 19-24 J. Pharmaceutical Science 70 No. 4 (1981) 387-389 Clinical Immunology and Immunopathology 14 (1979), 395-400. J. Histochemistry and Cytochemistry 29 No. 7 (1981) 870-873 J. Immunology 115 No. 3 (1975) 659-664

The file contains technical information submitted after the application was filed and not included in this specification

EP 0 016 552 B2

Description

This invention relates to the fractionation of heterogeneous populations of cells or the like to isolate a relatively homogeneous subpopulation of a specific cell type. More specifically the improvement of this invention relates to magnetic sorting of cells, bacteria, or viruses.

A general procedure for magnetic sorting of cells, bacteria, and viruses is disclosed in US--A--3,970,518. In that procedure, uncoated particles of a magnetic material, such as iron oxide, are contacted with a high concentration liquid dispersion of the selective antibody, and after sufficient antibody has adhered to the magnetic particles the coated particles are contacted with the mixed population to be fractionated, the select cell or the like binding to the magnetic particles and the bound cells are then separated magnetically from the remainder of the population. As a further step the select cells may be separated from the magnetic material, by the use of a cleaving agent solution and magnetic removal of the magnetic particles.

While there are literature reports describing the use of magnetic microspheres in cell sorting, there is no literature verification that uncoated magnetic particles can be made to effectively bind with antibodies. In the published procedures the particles of magnetic material are contained in microspheres formed from polymers, which can be chemically coupled to antibodies. See, for example: Molday et al, *Nature*, 268, 437 (1977), Kronick et al, *Science*, 200, 1074 (1978), and Antoine et al. *Immunochemistry*, 15, 443 (1978). These references describe magnetically-responsive microspheres formed from acrylate polymers, such as hydroxyethyl methacrylate, or polyacrylamide-agarose microspheres. Such microspheres can be chemically coupled to antibodies with glutaraldehyde or other dialdehyde. As described by the cited Molday (1977) and Kornick references, one procedure involves the chemical attachment of diaminoheptane spacer groups to the microspheres, which are then chemically linked to the antibodies by glutaraldehyde reaction. Such a reaction is for example disclosed in reference DE-OS 26 54 723. Although effective bounding of the antibodies can be obtained, such procedures are difficult since aggregation of microspheres can readily occur and the preparative procedure is time consuming. For example, the reaction to attach spacer groups may require from five to twelve hours of chemical reaction time, and subsequent dialysis to remove the excess reagent. The coupling of the antibodies may then require another twelve to twenty-four hours followed by dialysis to remove excess coupling agent. Further, such antibody reagents may not be used efficiently since an excess of the antibodies will usually need to be present during the chemical coupling.

Another disadvantage of magnetic particle or microsphere separation methods as described in the art is that the antibodies are attached to the microspheres in a random manner. Antigen-binding occurs through the Fab regions of the antibodies which are in the outer portions of the arms. With random attachment of the antibodies, one or both of the Fab arms may be unavailable for antigen-binding. Thus, an excess of antibody must be used to assure that the coated microspheres effectively bind to the antigens associated with the cells or other bodies being sorted.

The present invention utilizes staphylococcal Protein A to overcome the limitations of prior art magnetic sorting procedures, as described above. It is known that staphylococcal Protein A selectively binds to antibodies through the Fc region of the antibodies which is located in the tail portions of the antibodies remote from the Fab arms. See Forsgren et al, *J. Immunol.* 99, 19 (1967). Heretofore however, this property of Protein A has not been utilized to form magnetic microspheres. Protein A has been coupled to Sepharose beads (cross-linked agarose gels) to provide a column material with immunoglobulin-binding properties. The column may be used for affinity chromatography, for example, of the IgG fraction of serum. Such chromatographic column materials are commercially available.

Protein A has also been used in procedures for cell separation by density gradient centrifugation. See for example, Ghetie et al, *Scand J. Immunol.*, 4, 471 (1975). In a typical procedure, sheep erythrocytes are coated with Protein A by CrCl_3 coupling, and the coated erythrocytes are then contacted with mouse lymphocytes which have been previously reacted with antibodies to prepare the cell surfaces for binding to Protein A, thereby resulting in rosetting of the lymphocytes around the erythrocytes. The resulting rosetted cells are recovered by density gradient centrifugation.

Ghetie W. and Sjöquist J. disclosed in *J. Immunol.* 115(3) 659 (1975) a process whereby protein A adsorbed on the surface of a matrix (polystyrene) couples first with an antibody and then with a specific antigen. It is assumed that the protein A is attached to the antibody through the Fc arm whereby the antibody/ antigen couple with free Fc arms attaches itself to the protein A coated matrix.

In accordance with the present invention, as distinguished from prior art procedures, magnetically-responsive microspheres are prepared having Protein A associated with the surfaces thereof, and the resulting microspheres are first reacted with the select antibodies before the microspheres are used for cell separation. With the microspheres used in the method of this invention the antibodies are thereby arranged in oriented attachment on their outer surfaces with the Fab arms of the antibodies extending outwardly. The effectiveness of the microspheres for antigen binding and use in magnetic sorting procedures is thereby maximized. This greatly increases the efficiency with which the select antibodies may be used. Further, it eliminates the need for chemical coupling of the antibodies.

In a preferred embodiment, the microspheres are prepared by mixing Protein A with a polymer matrix material which does not mask the antibody-binding sites of the Protein A. The resulting microspheres having the Protein A in the outer surfaces thereof do not require chemical coupling of the Protein A to preformed microspheres. Albumin appears to be a particularly suitable matrix material for preparing such microspheres.

When the microspheres are formed from an aqueous admixture of albumin, Protein A, and magnetic particles, the Protein A is effectively available in the outer surfaces of the microspheres, in effect, forming surface layers on the microspheres with the Protein A in high concentration. The explanation for this result is not fully understood, but appears to relate to the wetting agent or surface tension properties of Protein A when dispersed in an aqueous solution in admixture with albumin.

The microspheres of the present invention are to be used in a method for separation of a select population of cells, bacteria, or viruses from a mixed population thereof. The microspheres containing magnetic particles are coated with a layer of antibodies which selectively bind to the select population. The coated microspheres are contacted with the mixed population, and the bound select population is magnetically separated from the rest of the mixed population. The method for preparing the inventive microspheres is characterized by modifying the surfaces of the microspheres prior to coating them with antibodies to provide staphylococcal Protein A distributed thereover in adherent relation to the microspheres. The microspheres are then contacted with antibodies which bind the Protein A and which also bind selectively to the select population. By this means the antibodies are arranged in oriented attachment on the surfaces of the microspheres with their Fab arms extending outwardly. Thereafter, the rest of the steps of the magnetic separation are carried out, as is known in the art. Preferably, the microspheres are formed of a polymer matrix material in admixture with the magnetic particles and Protein A, such as an albumin matrix material in an amount of 100 parts per 5 to 40 parts of Protein A. Alternatively, however, the Protein A may be chemically-bonded to the exterior surfaces of the microspheres to provide a Protein A coating thereon.

Where chemical coupling procedures are used, the microspheres may be formed from any matrix material which can be chemically coupled to Protein A, including albumin or other amino acid polymer, and synthetic polymers, such as acrylate polymers. For example, the microspheres may be formed from methyl methacrylate, hydroxyethyl methacrylate, methacrylic acid, ethylene glycol dimethacrylate, agarose polymers, polyacrylamide polymers, or mixtures of such polymers. Protein A may be directly coupled to solid support surfaces containing magnetically responsive materials by several procedures. See, for example, Molday et al, *J. Cell Biology*, 64, 75 (1975) Microspheres can be derivatized with either aminocaproic acid or diaminoheptane which provide extend functional groups for coupling proteins to insolubilized matrixes. Alternatively, with solid surfaces already containing functionally available groups (i.e. amino groups on albumin microspheres) a direct glutaraldehyde coupling of Protein A may be accomplished.

An alternate preferred procedure is to incorporate the Protein A in the microspheres by admixing it with the matrix material prior to the formation of the microspheres, and carrying out the preparation so that the Protein A is available in the outer surfaces of the microspheres. Suitable procedures for preparing such microspheres will therefore be described, but it should be understood that the present invention in its broad method aspect is not limited to the use of such preferred microspheres.

For use in the present invention, the Protein A can be prepared from *Staphylococcus aureus* by procedures described in the literature. See, for example, Forsgren et al, *J. Immun.*, 97, 822 (1966); and Konvall et al, *Immunochemistry*, 7, 124 (1970). Staphylococcal Protein A is also available from commercial sources, such as Pharmacia Fine Chemicals, Piscataway, New Jersey.

The preferred matrix material for forming the microspheres by admixture with Protein A is an amino acid polymer, such as albumin. Animal or human albumin may be used, for example, human serum albumin. Other water-soluble proteins can be used such as hemoglobin, or synthetic amino acid polymers including poly-L-lysine and poly-L-glutamic acid.

When the Protein A is premixed with the matrix polymer and the microspheres formed therefrom, sufficient Protein A should be included so that the outer surfaces of the microspheres will bind antibodies through the selective action of the Protein A. In general, the microspheres may contain from 2 to 40 parts by weight of Protein A per 100 parts of the matrix polymer such as albumin. Preferred proportions are from about 10 to 35 parts of the Protein A per 100 parts of the matrix polymer.

A sufficient amount of finely-divided particles of a magnetic material should also be included so that the microspheres are magnetically responsive. For example, the magnetic particles may be ferri- or ferro-magnetic compounds, such as magnetic iron oxides. Other useable magnetic materials in particulate form are disclosed in U.S.-A-3,970,518. A preferred magnetic material is magnetite (Fe_3O_4). Depending on the size of the microspheres, the magnetic particles may range in size from 10 to 2000 nm. The microspheres may contain from 10 to 150 parts by weight of the magnetic material per 100 parts of the matrix polymer.

The microspheres may range in size from 0.2 to 100 μm in diameter. Preferably however, the microspheres have an average size in the range from about 0.5 to 2.0 μm . With microspheres in this size range, it is preferred that the magnetic particles have diameters of not over 30 nm, such as an average size of about 10 nm.

The procedure previously published for preparing albumin microspheres can be used. Widder et al. *J. Pharm. Sci.*, 68, 79 (1979). The preferred procedure is the one described for the heat-stabilized microspheres. In general, an aqueous mixture is prepared for use in forming the microcapsules, the mixture containing the albumin or other hydrocolloid matrix polymer, Protein A, and the magnetic particles. The solid materials are dispersed in water and thoroughly mixed therewith, for example, using 20 to 40 parts of total solids per 100 parts of water. Sufficient water should be present to form an aqueous gel with the matrix hydrocolloid. In general the amount of water may range from 10 to 80 parts per 100 parts of total solids. The aqueous mix is then emulsified with an oil, such as a vegetable oil, the emulsification being carried out with vigorous agitation, for example, using sonication, to obtain a droplet dispersion of the aqueous mix in the vegetable oil having the

requisite droplet size to form the microspheres. Preferably, the emulsification is carried out at low temperatures such as temperatures in the range of 20 to 30°C. After the emulsion has been formed, the emulsion is added to a larger body of oil, which is preferably the same oil used to form the emulsion. In practice, cottonseed oil gives good results. To promote the separation of the water droplets, the emulsion can be added in small increments to the oil bath, such as by dropwise addition. Preferably, also, the addition is accompanied by rapid stirring of the oil into which the emulsion is being introduced.

For purpose of the present invention, the droplets may be heat-hardened to stabilize them and thereby provide the microspheres. This can be conveniently accomplished by using a heated oil bath, that is, by dispersing the emulsion into hot oil, such as oil at a temperature in the range of 70 to 160°C. The effect of heat stabilization on albumin microspheres is described in US--A--3,937,668.

After the heat-hardening, the prepared microspheres are separated from the oil. This may be accomplished by centrifugation or filtration, and the microspheres washed with a suitable organic solvent, such as diethyl ether, to remove the oil from the exterior surfaces of the microspheres. The microspheres are then ready for reaction with a specific antibody, such as an antibody prepared in rabbits. Such rabbit immunoglobulins which bind to Protein A include all subclasses of IgG. However, antibodies prepared from other sources can be used, providing they also bind to Protein A. Usually, the antibodies will be applied to the microspheres in aqueous suspension. The concentration of the antibodies may be low, since the Protein A will remove the antibodies from the treating solution even at low concentrations. As previously described, the binding is through the Fc region of the antibodies, thereby providing for an oriented attachment of the antibodies with the antigen-binding Fab arms extending outwardly from the outer surfaces of the microspheres. The microspheres are then ready for use in magnetic cell separation, as previously described in the literature.

The prepared microspheres of this invention for the use in the magnetic sorting method are further described and illustrated in the following specific examples. For conciseness of description, the examples use certain abbreviations, which have the following meanings:

SpA: Staphylococcal Protein A

FITC: fluorecein isothiocyanate

CRBC: chicken red blood cell

SRBC: sheep red blood cell

RBC: red blood cell

FCS: fetal calf serum

HBSS: Hank's balanced saline solution

EDC: carbodiimide: 1-cyclohexyl-3-(2-morpholinyl)-(4)-ethyl-carbodiimide methotoluene sulphonate)

Example I

Magnetic albumin microspheres, containing staphylococcal protein A (SpA) as part of the matrix were prepared by an emulsion polymerization method. A 0.5 ml aqueous suspension containing a total of 190 mg dry material was made consisting of 66 % human serum albumin, 19 % Fe₃O₄ (particles 15-20 nm) and 15 % SpA. To this, 60 ml of cottonseed oil was added and the emulsion was homogenized by sonication for one minute. The homogenate was added dropwise to 200 ml of constantly stirred cottonseed oil at 120 to 125°C for 10 minutes. The suspension was washed four times in diethyl ether by centrifugation for 15 minutes at 2000 xg and stored at 4°C until subsequent use. A sample of microspheres were coupled with FITC-conjugated rabbit IgG by incubation at 37°C for 20 minutes, and examined for surface fluorescence with a fluorescent microscope. The intensity and apparent uniform distribution of fluorescence indicated that SpA was oriented on the microsphere surface in a manner that allowed IgG molecules to interact with the Fc binding sites on the SpA.

Example II

Microspheres prepared as described in Example I were used to separate CRBC from suspensions containing both CRBC and SRBC. Aliquots of CRBC and SRBC were labeled with ⁵¹Cr in order to assess extent of separation as well as cell integrity. Labeling of CRBC was accomplished by incubating 1x10⁸ CRBC suspended in 0.2 ml Hanks balanced salt solution (HBSS) containing 2.5 % heat inactivated fetal calf serum (FCS) with 100 μCi Na₂⁵¹CrO₄ (1 mCi/ml) for 90 minutes at 37°C. SRBC were labeled by similar treatment with the exception of overnight incubation at 37°C. Antibody was coupled to the microspheres by incubating 0.5 mg of the microspheres suspended in 0.2 ml of 0.9 % NaCl solution containing 0.1 % Tween 80 (saline-Tween 80) with either 0.5 mg rabbit anti-chicken RBC (IgG) fraction) or 0.5 mg normal rabbit IgG for 45 minutes at 37°C. Unbound IgG was removed by centrifugation with excess saline-Tween 80 at 1500 xg for two minutes at 4°C. Microspheres were then resuspended in 0.2 ml saline-Tween 80 by briefly sonicating in an ultrasonic waterbath. To this suspension, a mixture of 1x10⁶ CRBC and 1x10⁶ SRBC in 0.2 ml of HBSS was added. The cells were then incubated with the IgG-coated microspheres for 30 minutes at 37°C with mild agitation. Cells bearing adherent magnetic microspheres were removed from suspension by applying a 4000 gauss (gradient--

1500 gauss/cm) bar magnet to the side of each test tube for one minute. Both supernatant and pellet fractions were counted in a Beckman Model 8000 gamma counter for ^{51}Cr . Control labeled cells, incubated in saline-Tween 80, were counted for ^{51}Cr to assess spontaneous release.

Based on ^{51}Cr counts, it was found that when 1×10^6 ^{51}Cr CRBC in combination with 1×10^6 SRBC were incubated with 0.5 mg microspheres bearing anti-CRBC antibodies, 97.8 % of the labeled cells were magnetically removed from suspension. Hemocytometer counts of erythrocytes in the supernatant revealed only 0.26 % residual CRBC among the remaining SRBC. Using this method of cell separation, a population of SRBC which was 97-99 % homogeneous was generated with 90.5 % recovery of the starting SRBC mass. The nonspecific adherence of ^{51}Cr CRBC was tested while using microspheres bearing anti-SRBC and normal rabbit IgG respectively and found to be < 10 %

Example III

Microspheres prepared as described in Example I were used to fractionate Lewis rat splenocytes. Based on the presence or absence of surface immunoglobulins, it is possible to distinguish between thymus-derived T lymphocytes and bone-marrow derived B lymphocytes. Normal non-IgG bearing splenocytes, considered to be predominantly T lymphocytes, were purified by incubating splenocytes with microspheres containing rabbit anti-rat IgG.

A cellular suspension of spleen cells was obtained by teasing rat spleen on a metal screen in HBSS with 10 % heat inactivated FCS. The cells were washed three times and over-layered on Ficoll-Hypaque (specific gravity 1.072). The gradient was then centrifuged at 1200 xg for 25 minutes at 25°C, to eliminate dead cells and red blood cells. The resultant interface band was removed and assessed for viability by trypan blue dye exclusion. The number of IgG bearing cells was determined by incubating the cells at 37°C with FITC conjugated rabbit anti-rat IgG and counting the number of fluorescent labeled cells. Rabbit anti-rat IgG, normal rabbit IgG and rabbit anti-chicken RBC were coupled to 0.5 mg the SpA microspheres (0.5 mg IgG/0.5 mg microspheres). Splenocytes (2×10^6) suspended in HBSS with 2.5 % heat inactivated FCS were added to the 0.5 mg microspheres. In order to minimize the capping phenomenon and maintain viability, reaction mixtures were incubated for 2.5 hours at 4°C. Cells with adherent microspheres were separated magnetically as described in Example II, and resultant supernatants were analyzed for total cell count, viability, and fluorescence. The results are summarized in Table A.

Viability of unfractionated cells after centrifugation in Ficoll-Hypaque was 96 % Supernatant cell viability after magnetic separation was 93 %, demonstrating a minimal loss of viability. Between 47 to 51 % of unfractionated splenocytes were IgG-bearing cells as determined by fluorescence microscopy. However, after magnetic separation of splenocytes in the experimental group, only 0.5 % of the supernatant cells had detectable IgG on their surface, showing a highly enriched population of non-IgG-bearing lymphocytes.

Antibody specificity was verified by demonstrating negligible depletion of IgG-bearing cells following incubation of splenocytes with microspheres containing either normal rabbit IgG or anti-CRBC. Rat thymocytes, normally containing 4 to 6 % IgG bearing lymphocytes, were totally depleted of these cells after incubation with microspheres coupled with anti-rat IgG.

The sensitivity of the system was tested by serial dilutions of microspheres bearing rabbit anti-chicken RBC with the addition of 1×10^6 ^{51}Cr CRBC at each dilution. Incubation of microspheres and cells was carried out for 30 minutes at 37°C. Cells with adherent microspheres were magnetically removed and both pellet and supernatant fractions counted for ^{51}Cr . Percent CRBC bound to the microspheres was linearly related to the amount of microspheres present until microsphere saturation occurred. No less than 99 % binding of 1×10^6 CRBC was observed when ≥ 104 μg of microspheres were used.

TABLE A

Separation of rat T and B lymphoid cells using antibodies coupled to SpA-bearing magnetic microspheres (Result represent two different experiments done in triplicate and expressed as mean \pm S.D)

	Antibody coupled to microspheres	Type of cell suspension (1)	% of total cells bound to microspheres	% of IgG bearing cells remaining in supernatant after magnetic separation(2)
5	Rabbit anti-rat IgG	Splenocytes	50 ± 0.5	0.5
	Rabbit anti-rat IgG	Thymocytes	15 ± 1	0
	Normal rabbit IgG	Splenocytes	10 ± 0.3	47.4 ± 1.2(3)
	Rabbit anti-CRBC	Splenocytes	6.5 ± 2	44 ± 1.8(3)

Footnotes to Table A

10 (1) 2×10^6 cells/0.2 ml HBSS + 2.5 % FCS, incubated with 0.5 mg microspheres bearing antibody as indicated. The reaction mixture was incubated for 2.5 hours at 4°C.

(2) The percent of IgG-bearing cells not removed by magnetic microspheres was determined by incubating supernatant cells with 0.1 ml FITC conjugated rabbit anti-rat IgG for 20 minutes at 37°C. The amount of contaminant IgG-bearing cells was determined by fluorescence microscopy. In addition, 95.3 % of the
15 expected non-IgG-bearing splenocytes was found in the supernatant as determined by triplicate counts in a hemocytometer of non-fluorescent cells

(3) Between 44 to 51 % starting splenocytes are IgG-bearing cells as determined by fluorescence microscopy, and 4 to 6 % of rat thymocytes are IgG-bearing cells as determined by the same method.

20

Example IV

25 Magnetic albumin microspheres were prepared as described in Example I, except that Protein A was omitted. The amount of albumin was correspondingly increased so that the dry material used to form the microspheres was 81 % albumin and 19 % Fe_3O_4 . Protein A can be applied to the microspheres thus formed as described in Example V.

30

Example V

5 mg/ml Protein A in HBSS is prepared as a starting solution For the one step aqueous carbodiimide coupling 10 mg of EDC is added to 20 mg of either an acrylate polymer microsphere matrix prederivatized with
35 ϵ -aminocaproic acid, or 20 mg of albumin microspheres suspended in 10 mls of the starting Protein A solution and allowed to react for 4 hrs at 4°C with vigorous stirring. The coupling reaction is then terminated by addition of 0.4 ml of 0.2 M glycine solution pH 7.9. Unbound Protein A and unreacted EDC is removed by washing 4x in HBSS. The Protein A coated microspheres are then coupled to appropriate antisera by incubation of 2 mg of
40 microspheres with 1 ml of antiserum at 37°C for 10 mins with slight agitation The alternative coupling agent is glutaraldehyde 1.25 % solution which is added to 10 mls of HBSS solution (pH 7.4) containing 50 mg Protein A and 20 mg of either albumin microspheres or 20 mg of diaminoheptane derivatized acrylate microspheres, allowed to react for 2 hrs at 37°C with slight agitation. Unreacted Protein A and excess glutaraldehyde is removed by centrifugation washing 4x with HBSS. Antibody is attached to microspheres as described above in
45 prior examples.

Claims

50 1. Microspheres for magnetically sorting of cells, bacteria, or viruses, comprising microspheres formed from an amino acid polymer matrix material in admixture with staphylococcal Protein A, and being of a size from 0.2 to 100 μm diameter, said microspheres containing from 2 to 40 parts by weight of said Protein A per 100 parts of said amino acid polymer and said Protein A being present in the exterior surfaces of said microspheres for antibody binding, said microspheres also containing magnetic particles of a size from 10 to 2000 nm and in an
55 amount sufficient to make said microspheres magnetically-responsive.

2. The microspheres of claim 1 in which said amino acid polymer is albumin.

3. The microspheres of claim 1 or claim 2 in which said Protein A is present in an amount of from 10 to 30 parts by weight per 100 parts of said amino acid polymer.

4. Microspheres for magnetically sorting of cells, bacteria, or viruses, comprising microspheres formed from
60 an aqueous mixture of albumin, staphylococcal Protein A, and magnetic particles, said microspheres having an average diameter of from 0.5 to 2.0 μm , and containing from 2 to 40 parts of said Protein A per 100 parts of said albumin and said Protein A being present in the exterior surfaces of said microspheres for antibody binding, said magnetic particles being of a size not over 30 nm and being present in an amount sufficient to make said microspheres magnetically-responsive.

65 5. The microspheres of claim 4 in which said magnetic particles are Fe_3O_4 and are present in an amount of

from 10 to 150 parts by weight per 100 parts of said albumin.

6. The microspheres of claim 4 or claim 5 in which said Protein A is present in an amount of from 10 to 35 parts by weight per 100 parts of said albumin.

5 7. A method for the separation of a select population of cells, bacteria or viruses from a mixed population thereof, in which microspheres defined in any of claims 1 to 6 are employed, using an antibody bound to the microspheres as a means for sorting the cells, bacteria or viruses from the mixed population thereof.

10 Revendications

1. Microsphères pour le triage magnétique des cellules, bactéries et virus comprenant des microsphères formées en une matière de matrice qui est polymère d'acide aminé en mélange avec de la Protéine A staphylococcique et qui ont une taille comprise entre 0,2 et 100 µm de diamètre, lesdites microsphères
15 contenant de 2 à 40 parties en poids de ladite Protéine A pour 100 parties dudit polymère d'acide aminé et ladite protéine A étant présente dans les surfaces extérieures desdites microsphères pour la fixation d'anticorps, lesdites microsphères contenant également des particules magnétiques ayant une taille comprise entre 10 et 2000 nm et en quantité suffisante pour rendre lesdites microsphères magnétiquement sensibles.

2. Les microsphères de la revendication 1, dans lesquelles le polymère d'acide aminé est de l'albumine.

20 3. Les microsphères de la revendication 1 ou de la revendication 2 dans lesquelles ladite Protéine A est présente dans une proportion comprise entre 10 et 30 parties en poids pour 100 parties dudit polymère d'acide aminé.

4. Microsphères pour le triage magnétique de cellules, bactéries ou virus comprenant des microsphères formées à partir d'un mélange aqueux d'albumine, de Protéine A staphylococcique et de particules
25 magnétiques, lesdites microsphères ayant un diamètre moyen compris entre 0,5 et 2,0 µm et contenant de 2 à 40 parties de ladite Protéine A pour 100 parties de ladite albumine et ladite Protéine A étant présente dans les surfaces extérieures desdites microsphères pour la fixation d'anticorps, lesdites particules magnétiques ayant une taille inférieure à 30 nm et étant présentes en une quantité suffisante pour rendre lesdites microsphères magnétiquement sensibles.

30 5. Les microsphères de la revendication 4 dans lesquelles lesdites particules magnétiques sont du Fe₃O₄ et sont présentes dans une proportion comprise entre 10 à 150 parties en poids pour 100 parties de ladite albumine.

6. Les microsphères de la revendication 4 ou de la revendication 5 dans lesquelles ladite Protéine A est présente dans une proportion comprise entre 10 et 35 parties en poids, pour 100 parties de ladite albumine.

35 7. Procédé pour la séparation d'une population choisie de cellules bactériennes ou virus d'une population mélangée de tels corps, suivant lequel des microsphères telles que définies dans l'une des revendications 1 à 6 sont employées, en utilisant un anticorps lié aux microsphères comme moyen de triage des cellules, des bactéries ou des virus de la population mélangée.

40 Patentansprüche

1. Mikrosphären zum magnetischen Sortieren von Zellen, Bakterien oder Viren, enthaltend Mikrosphären, gebildet aus einem Aminosäurepolymaterial, im Gemisch mit von Staphylococcen stammendem
45 Protein A und mit einer Größe von 0,2 bis 100 µm Durchmesser, wobei die Mikrosphären 2 bis 40 Gew.-Teile des Proteins A pro 100 Teile des Aminosäurepolymeren enthalten und das Protein A in den äußeren Oberflächen der Mikrosphären zur Bindung von Antikörpern vorhanden ist, wobei die Mikrosphären auch magnetische Teilchen mit einer Größe von 10 bis 2000 nm und in ausreichender Menge enthalten, um die Mikrosphären
50 magnetisch reagierend zu machen.

2. Mikrosphären nach Anspruch 1, in denen das Aminosäurepolymere Albumin ist.

3. Mikrosphären nach Anspruch 1 oder 2, in denen das Protein A in einer Menge von 10 bis 30 Gew.-Teilen pro 100 Teile des Aminosäurepolymeren vorhanden ist.

4. Mikrosphären zum magnetischen Sortieren von Zellen, Bakterien oder Viren, enthaltend Mikrosphären, gebildet aus einem wässrigen Gemisch von Albumin, von Staphylococcen stammendem Protein A und
55 magnetischen Teilchen, wobei die Mikrosphären einen durchschnittlichen Durchmesser von 0,5 bis 2,0 µm aufweisen und 2 bis 40 Teile des Proteins A pro 100 Teile des Albumins enthalten und das Protein A in den äußeren Oberflächen der Mikrosphären zur Antikörperbindung vorhanden ist, wobei die magnetischen Teilchen eine Größe von nicht über 30 nm aufweisen und in ausreichender Menge vorhanden sind, um die
60 Mikrosphären magnetisch reagierend zu machen.

5. Mikrosphären nach Anspruch 4, in denen die magnetischen Teilchen Fe₃O₄ sind und in einer Menge von 10 bis 150 Gew.-Teilen pro 100 Teile des genannten Albumins vorhanden sind.

6. Mikrosphären nach Anspruch 4 oder 5, in denen das Protein A in einer Menge von 10 bis 35 Gew.-Teilen pro 100 Teile des genannten Albumins vorhanden sind.

65 7. Ein Verfahren zum Abtrennen einer ausgewählten Population von Zellen, Bakterien oder Viren aus einer

0 016 552

: gemischten Population aus diesen, bei der die Mikrosphären, wie in einem der Ansprüche 1 bis 6 definiert, angewendet werden, unter Verwendung eines an die Mikrosphären gebundenen Antikörpers als Mittel zum Sortieren der Zellen, Bakterien oder Viren aus einer gemischten Population aus diesen.

5

10

15

20

25

30

35

40

45

50

55

60

65